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PATENT APPLICATION

TRAC1: MODULATORS OF LYMPHOCYTE ACTIVATION

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CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority to USSN 60/282,432, filed April 6, 2001, herein incorporated by reference in its entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

FIELD OF THE INVENTION

The present invention relates to regulation of T lymphocyte activation. More particularly, the present invention is directed to nucleic acids encoding TRAC1, which is involved in modulation of T lymphocyte activation and TCR signaling. The invention further relates to methods for identifying and using agents, including small organic molecules, peptides, circular peptides, antibodies, lipids, antisense nucleic acids, and ribozymes, that modulate lymphocyte activation and TCR signaling via modulation of TRAC1; as well as to the use of expression profiles and compositions in diagnosis and therapy related to lymphocyte activation and suppression.

BACKGROUND OF THE INVENTION

The immune response includes both a cellular and a humoral response. The cellular response is mediate largely by T lymphocytes (alternatively and equivalently referred to herein as T cells), while the humoral response is mediated by B lymphocytes (alternatively and equivalently referred to herein as B cells). Lymphocytes play a number of crucial roles in immune responses, including direct killing of virus-infected cells, cytokine and antibody production, and facilitation of B cell responses. Lymphocytes are also involved in acute and chronic inflammatory disease; asthma; allergies; autoimmune diseases such as scleroderma, pernicious anemia, multiple sclerosis, myasthenia gravis, IDDM, rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease; and organ and tissue transplant disease, e.g., graft vs. host disease.

B lymphocytes produce and secrete antibodies in response to the concerted presentation of antigen and MHC class II molecules on the surface of antigen presenting

cells. Antigen presentation initiates B cell activation through the B cell receptor (BCR) at the B cell surface. Signal transduction from the BCR leads to B cell activation and changes in B cell gene expression, physiology, and function, including secretion of antibodies.

T cells do not produce antibodies, but many subtypes of T cells produce co-stimulatory molecules that augment antibody production by B cells during the humoral immune response. In addition, many T cells engulf and destroy cells or agents that are recognized by cell surface receptors. Engagement of the cell surface T cell receptor (TCR) initiates T cell activation. Signal transduction from the TCR leads to T cell activation and changes in T cell gene expression, physiology, and function, including the secretion of cytokines.

Identifying ligands, receptors, and signaling proteins downstream of TCR, as well as BCR, activation is important for developing therapeutic regents to inhibit immune response in inflammatory disease, autoimmune disease, cancer, and organ transplant, as well as to activate immune response in immunocompromised subjects, and in patients with infectious disease and cancer (*see, e.g.*, Rogge *et al., Nature Genetics* 25:96-101 (2000); US Patent Nos. 5,518,911; 5,605,825; 5,698,428; 5,698,445; 6,013,464; and 6,048,706). In addition, identification of the function of a protein with a previously unknown function allows the development of therapeutic agents for disease associated with the protein.

SUMMARY OF THE INVENTION

The present invention provides nucleic acids encoding TRAC1, which is a member of the ring finger protein family and an E3 ubiquitin ligase involved, e.g., in ubiquitination and subsequent protein degradation, cellular proliferation and cancer, inflammation, autoimmune disease, developmental abonormalities, neurodegenerative disease, and modulation of T lymphocyte activation and TCR signaling. TRAC1 binds to E2 ubiquitin conjugating enzymes such as UbcH5, 7, and 8. The invention therefore provides methods of screening for compounds, e.g., small organic molecules, antibodies, peptides (such as TRAC1 ring finger or ligase domain fragments), circular peptides, lipids, antisense molecules, RNAi (double stranded inhibitory RNA) and ribozymes, that are capable of modulating ubiquitination and/or lymphocyte activation, including TCR signaling, e.g., either activating or inhibiting T lymphocytes. Therapeutic and diagnostic methods and reagents are also provided.

In one aspect of the invention, nucleic acids encoding TRAC1 are provided. In another aspect, the present invention provides nucleic acids, such as probes, antisense oligonucleotides, and ribozymes, that hybridize to a gene encoding a TRAC1 protein. In another aspect, the invention provides expression vectors and host cells comprising TRAC1-encoding nucleic acids. In another aspect, the present invention provides TRAC1 protein, and antibodies thereto.

In another aspect, the present invention provides a method for identifying a compound that modulates T lymphocyte activation, the method comprising the steps of: (i) contacting the compound with a TRAC1 polypeptide or a fragment thereof, the polypeptide or fragment thereof encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a polypeptide having an amino acid sequence of SEQ ID NO:1; and (ii) determining the functional effect of the compound upon the TRAC1 polypeptide.

In another aspect, the present invention provides a method for identifying a compound that modulates T lymphocyte activation, the method comprising the steps of: (i) contacting a T cell comprising a TRAC1 polypeptide or fragment thereof with the compound, the TRAC1 polypeptide or fragment thereof encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a polypeptide having an amino acid sequence of SEQ ID NO:1; and (ii) determining the chemical or phenotypic effect of the compound upon the cell comprising the TRAC1 polypeptide or fragment thereof, thereby identifying a compound that modulates T lymphocyte activation.

In another aspect, the present invention provides a method for identifying a compound that modulates T lymphocyte activation, the method comprising the steps of: (i) contacting the compound with a TRAC1 polypeptide or a fragment thereof, the TRAC1 polypeptide or fragment thereof encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a polypeptide having an amino acid sequence of SEQ ID NO:1; (ii) determining the physical effect of the compound upon the TRAC1 polypeptide; and (iii) determining the chemical or phenotypic effect of the compound upon a cell comprising the TRAC1 polypeptide or fragment thereof, thereby identifying a compound that modulates T lymphocyte activation.

In one embodiment, the functional effect is measured *in vitro*. In one embodiment, the functional effect is a physical effect. In another embodiment, the functional effect is a chemical or phenotypic effect.

In one embodiment, the host cell is primary T lymphocyte or a cultured T lymphocyte, e.g., a Jurkat cell.

In another embodiment, the chemical or phenotypic effect is determined by measuring CD69 expression, intracellular Ca^{2+} mobilization, Ca^{2+} influx, ligase activity (e.g., ubiquitin ligase activity or ubiquitin ligase-like activity), or lymphocyte proliferation.

In another embodiment, modulation is inhibition of T lymphocyte activation.

In another embodiment, the polypeptide is recombinant. In another embodiment, the TRAC1 polypeptide comprises an amino acid sequence of SEQ ID NO:1. In another embodiment, the TRAC1 polypeptide is encoded by a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2.

In another embodiment, the compound is an antibody, antisense molecule, small organic molecule, peptide, a lipid, a ribozyme, RNAi, or a circular peptide.

In one aspect, the present invention provides a method for identifying a compound capable of interfering with binding of an TRAC1 polypeptide or fragment thereof, the method comprising the steps of: (i) combining an TRAC1 polypeptide or fragment thereof with an E2 ubiquitin-conjugating enzyme polypeptide and the compound, wherein the TRAC1 polypeptide or fragment thereof is encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a polypeptide having an amino acid sequence of SEQ ID NO:2; and (ii) determining the binding of the TRAC1 polypeptide or fragment thereof to the E2 ubiquitin-conjugating enzyme polypeptide.

In one embodiment, the TRAC1 polypeptide or fragment thereof has ligase activity.

In another embodiment, the E2 ubiquitin-conjugating enzyme polypeptide is selected from the group consisting of Ubc5, Ubc7, and Ubc8.

In one embodiment, the TRAC1 polypeptide or fragment thereof and the E2 ubiquitin-conjugating enzyme polypeptide are combined first.

In one embodiment, the reaction is performed *in vitro*.

In another embodiment, the TRAC1 polypeptide or fragment thereof and the E2 ubiquitin-conjugating enzyme polypeptide are expressed in a cell, e.g., a yeast cell.

In one embodiment, the TRAC1 polypeptide and/or the E2 polypeptide are recombinant. In another embodiment, the TRAC1 polypeptide or fragment thereof is fused to a heterologous polypeptide.

In another embodiment, the binding of the TRAC1 polypeptide or fragment thereof to the E2 ubiquitin-conjugating enzyme polypeptide is determined by measuring reporter gene expression.

In one aspect, the present invention provides a complex comprising a TRAC1 polypeptide bound to an E2 ubiquitin-conjugating enzyme polypeptide.

In another aspect, the present invention provides a method of modulating T lymphocyte activation in a subject, the method comprising the step of administering to the subject a therapeutically effective amount of a compound identified using the methods described above.

In one embodiment, the subject is a human.

In another aspect, the present invention provides a method of modulating T lymphocyte activation in a subject, the method comprising the step of administering to the subject a therapeutically effective amount of a TRAC1 polypeptide, the polypeptide encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a polypeptide having an amino acid sequence of SEQ ID NO:1.

In another aspect, the present invention provides a method of modulating T lymphocyte activation in a subject, the method comprising the step of administering to the subject a therapeutically effective amount of a nucleic acid encoding a TRAC1 polypeptide, wherein the nucleic acid hybridizes under stringent conditions to a nucleic acid encoding a polypeptide having an amino acid sequence of SEQ ID NO:1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Figure 1A shows an amino acid of human wild-type TRAC1 (SEQ ID NO:1); Figure 1B shows a cDNA sequence of human wild-type TRAC1 (SEQ ID NO:2); Figure 1C shows a second, slightly shorter cDNA sequence of human wild type TRAC1; Figure 1D shows a cDNA sequence encoding a truncated version of TRAC1 (SEQ ID NO:4; nucleotides 127-891 of SEQ ID NO:3); Figure 1E shows a genomic sequence of human wild type TRAC1 (SEQ ID NO:5); and Figure 1F shows a cDNA (SEQ ID NO:6) and amino acid sequence (SEQ ID NO:7) for mouse wild type TRAC1.

Figure 2 shows that mutant TRAC1 (FLJ20456) inhibited CD69 expression in Jurkat cells.

Figure 3 shows that wild-type or full length TRAC1 (FLJ20456) did not inhibit CD69 expression in Jurkat cells.

Figure 4 shows that mutant TRAC1 (FLJ20456) inhibited T cell but not B cell activation.

Figure 5 shows that TRAC1 (FLJ20456) is strongly expressed in lymphoid and hematopoietic cells.

Figure 6 shows that TRAC1 is predominantly expressed in resting T lymphocytes.

Figure 7 shows that TRAC1 (FLJ20456) is similar to two sequences with ring domains.

Figure 8 shows an alignment of ring finger domains.

Figure 9 shows ring finger vs. zinc finger domains.

Figure 10A shows ubiquitin pathway components. Figure 10B shows enzymology of ubiquitination.

Figure 11A shows a substrate-independent assay for ligase catalysis. Figure 11B shows bacterially expressed TRAC1 for ligase activity assay.

Figure 12A shows that TRAC1 exhibits E3 ubiquitin ligase activity. Figure 12B shows that the ring domain is required for TRAC1 ligase activity.

Figure 13A shows point mutations in conserved cysteine residues of the TRAC1 ring finger domain. Figure 13B shows point mutations in the conserved cysteine residues of the TRAC1 ring finger domain disrupt ligase activity.

Figure 14 shows expression of TRAC1 mRNA is ~ 8-fold higher in PBMC than in Jurkat cells.

Figure 15 shows that C-terminally truncated TRAC1 blocks TCR-induced calcium influx.

Figure 16 shows that an intact TRAC1 ring finger domain is required for inhibition of TCR-induced CD69 up-regulation.

Figure 17 provides a summary of functional effects by different TRAC1 constructs.

Figure 18 shows that TRAC1 binds to ubiquitin-conjugating enzymes (E2) UbcH5 and UbcH7 *in vitro*.

Figure 19 shows one model of TRAC1 regulation of T cell activation and ubiquitination, without being bound to any one theory.

DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

TRAC1, a protein from the "ring finger" family has been functionally identified as a protein involved ubiquitination as an E3 ubiquitin ligase, and in regulating T lymphocyte activation and TCR signaling. TRAC1 (also referred to as "FLJ20456") was identified in a functional genetic screen using CD69 as a readout of T cell activation. A nucleic acid encoding a mutant variant of human TRAC1 (SEQ ID NO:4) was recovered as an inhibitor of T cell activation-induced CD69 expression. Mutant TRAC1 expression in Jurkat cells results in inhibition of TCR induced CD69 upregulation and inhibition of calcium influx. The present application therefore also demonstrates that TRAC1 is involved in the TCR signaling pathway. These results indicate that TRAC1 modulators can be used for inhibition of TCR signaling and lymphocyte activation.

The present invention for the first time also identifies the function of TRAC1 as an E3 ubiquitin ligase (*see, e.g.*, Weissman, *Nat. Rev. Mol. Cell Biol.* 2:169-178 (2001)). The invention also identifies ligands for TRAC1, E2 ubiquitin conjugating enzymes such as UbcH5, 7, and 8. The ring domain is required for TRAC1 ligase activity. In addition, ligase activity of the C-terminally truncated TRAC1 mutant is required for inhibition of T cell activation. As the ring finger and ligase domains are likely involved in CD69 modulation, T cell activation and ubiquitination, in one embodiment TRAC1 or a fragment of TRAC1 such as the ring domain or ligase domain can be used *in vitro* in high throughput binding or ligase activity assays to identify modulators of TRAC1.

About 250 ring finger containing proteins have been identified in the human genome. Ring finger domains promote protein-protein interactions between a wide variety of proteins. Generally, the binding of zinc and formation of a right ring structure is required for protein-protein interactions and biological activity. Several ring finger proteins have been shown to function in the ubiquitination pathway. The majority of protein degradation occurs through the ubiquitin/proteosome system. Ubiquitin (E1) is a highly conserved 76 kDa protein. Multi-ubiquitin chains are attached to proteins and used to target the proteins to the proteosome for degradation. Ubiquitin ligases are a large family of enzymes, many of which contain ring finger domains. Human TRAC1 protein

is a novel ubiquitin ligase and is predominantly expressed in lymphoid cells, with highest expression in resting lymphoid cells.

The present invention identifies TRAC1 as a member of the TCR signaling pathway. The present invention also identified TRAC1 as a ubiquitin ligase. The present invention, therefore, has functionally identified TRAC1 as therapeutic drug targets for compounds, e.g., to treat diseases such as cancer, inflammation, and diseases related to ubiquitination, and as a drug target for compounds, e.g., to treat diseases related to T cell activation. For example, TRAC1 is a drug target for compounds that suppress or activate T lymphocyte activation, preferably T lymphocyte activation, e.g., for the treatment of diseases in which modulation of the immune response is desired, e.g., for treating diseases related to T lymphocyte activation, such as delayed type hypersensitivity reactions; asthma; allergies; cancer; autoimmune diseases such as scleroderma, pernicious anemia, multiple sclerosis, myasthenia gravis, IDDM, rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease; and conditions related to organ and tissue transplant, such as graft vs. host disease; and acute and chronic inflammation; as well as in diseases in which activation of the immune response is desired, e.g., in immunocompromised subjects, e.g., due to HIV infection or cancer; and in infectious disease caused by viral, fungal, protozoal, and bacterial infections. Preferably, modulators are compounds that inhibit TRAC1 and thereby inhibit T cell activation.

DEFINITIONS

By "disorder associated with T lymphocyte activation" or "disease associated with lymphocyte activation" herein is meant a disease state which is marked by either an excess or a deficit of T cell activation, including TCR signaling. For example, lymphocyte activation disorders associated with increased activation include, but are not limited to, acute and chronic inflammation, cancer, asthma, allergies , autoimmune disease and transplant rejection. Pathological states for which it may be desirable to increase lymphocyte activation include HIV infection that results in immunocompromise, cancer, and infectious disease such as viral, fungal, protozoal, and bacterial infections.

By "disorder associated with ubiquitination" or "disease associated with ubiquitination" herein is meant a disease state that is marked by a change in ubiquitination, e.g., changes in protein degradation leading to changes in aoptosis, regulation of G1 cell cycle progression, cellular signaling, the level of certain proteins,

signal transduction, and cellular proliferation, e.g., inflammation and cancer. Disorder associated with ubiquitination also include disorders associated with ubiquitin-like ligases, such as sumo, ruby, and nedd, which are ubiquitin homologs that are involved in protein modification.

The terms "TRAC1" protein or fragment thereof, or a nucleic acid encoding "TRAC1" or a fragment thereof refer to nucleic acids and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to an amino acid sequence encoded by a TRAC1 nucleic acid (SEQ ID NO:2, 3 or 5) or amino acid sequence of a TRAC1 protein (SEQ ID NO:1); (2) specifically bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising an amino acid sequence of a TRAC1 protein (SEQ IS NO:1), immunogenic fragments thereof, and conservatively modified variants thereof; (3) specifically hybridize under stringent hybridization conditions to an anti-sense strand corresponding to a nucleic acid sequence encoding a TRAC1 protein (SEQ ID NO:1), and conservatively modified variants thereof; (4) have a nucleic acid sequence that has greater than about 60% sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or higher nucleotide sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more nucleotides, to a TRAC1 nucleic acid (SEQ ID NO:2, 3, or 5).

A TRAC1 polynucleotide or polypeptide sequence is typically from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; cow, pig, horse, sheep, or any mammal. The nucleic acids and proteins of the invention include both naturally occurring or recombinant molecules. Exemplary nucleic acid and protein sequences for human TRAC1 are provided by GenBank Accession Nos. NM_017831, NP_060301.1, and EST B11666910 (see also Figure 1). Exemplary mouse wild type TRAC1 nucleotide and amino acid sequence are also provided (see Figure 1). As described herein, wild-type TRAC1 proteins have ubiquitin ligase activity and/or ubiquitin ligase-like activity.

"Ubiquitin ligase activity" refers to a ubiquitination enzyme capable of catalyzing the covalent binding of ubiquitin to another protein. *See, e.g.,* WO 01/75145 for assays for ubiquitin ligase activity.

Ubiquitin-like ligase activity refers to the ligase activity of ubiquitin homologs such as sumo, ruby, and nedd (*see, e.g., Nat. Rev. Mol. Cell. Biol.* volume 2 (2001)). These ubiqutin homologs are involved in protein modification and localization, and in some cases prevent ubiquitination and subsequent protein degradation.

"E2 ubiquitin conjugating enzyme" refers to a component of the ubiquitin ligation cascade such as Ubc5, 7, and 8. Many species of E2 have been identified, some of which act in preferred pairs with specific E3 enzymes to confer specificity for different target proteins. While the nomenclature for E2 is not standardized across species, investigators in the field have addressed this issue and the skilled artisan can readily identify various E2 proteins as well as orthologs (*see, e.g.*, Weissman, *Nature Reviews* 2:169-178 (2001); Haas & Sipemann, *FASEB J.* 11:1257-1268 (1997)).

The phrase "functional effects" in the context of assays for testing compounds that modulate activity of a TRAC1 protein includes the determination of a parameter that is indirectly or directly under the influence of TRAC1, e.g., an indirect, chemical or phenotypic effect such as inhibition of T lymphocyte activation represented by a change in expression of a cell surface marker or cytokine production upon TCR stimulation, or changes in cellular proliferation or apoptosis, ligase activity (ubiquitin ligase activity or ubiquitin-like ligase activity), or TCR signal transduction leading to increases in intracellular calcium or calcium influx; or, e.g., a direct, physical effect such as ligand/substrate binding or inhibition of ligand/substrate binding to TRAC1 (e.g., binding of E2 ubiquitin conjugating enzymes such as UbcH5, 7, and 8) or a TRAC1 domain such as the ring finger or ligase domain. A functional effect therefore includes ligand/substrate binding activity, the ability of cells to proliferate, apoptosis, gene expression in cells undergoing activation, ligase activity (ubiquitin ligase activity or ubiquitin-like ligase activity), expression of cell surface molecules such as CD69, TCR signal transduction, including downstream effectors such as second messengers, intracellular calcium release and calcium influx, production of cytokines, and other characteristics of activated lymphocytes. "Functional effects" include *in vitro*, *in vivo*, and *ex vivo* activities.

By "determining the functional effect" is meant assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of TRAC1 protein, e.g., measuring physical and chemical or phenotypic effects. Such functional effects can be measured by any means known to those skilled in the art, e.g., changes in spectroscopic (e.g., fluorescence, absorbance, refractive index), hydrodynamic

(e.g., shape), chromatographic, or solubility properties for the protein; measuring inducible markers or transcriptional activation of the protein; measuring binding activity or binding assays, e.g. binding to antibodies; measuring changes in ligand binding affinity, either naturally occurring or synthetic; measuring cellular proliferation; measuring apoptosis; measuring cell surface marker expression, e.g., CD69; measuring cytokine; measurement of changes in protein levels for TRAC1-associated sequences; measurement of RNA stability; phosphorylation or dephosphorylation; ligase activity; TCR signal transduction and downstream effectors, e.g., receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP3, or intracellular Ca^{2+}); calcium influx; identification of downstream or reporter gene expression (CAT, luciferase, β-gal, GFP and the like), e.g., via chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, and ligand binding assays.

"Inhibitors", "activators", and "modulators" of TRAC1 polynucleotide and polypeptide sequences are used to refer to activating, inhibitory, or modulating molecules identified using *in vitro* and *in vivo* assays of TRAC1 polynucleotide and polypeptide sequences. Inhibitors are compounds that, e.g., bind to, partially or totally block activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity or expression of TRAC1 proteins, e.g., antagonists. "Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate TRAC1 protein activity. Inhibitors, activators, or modulators also include genetically modified versions of TRAC1 proteins, e.g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, peptides, cyclic peptides, nucleic acids, antibodies, antisense molecules, ribozymes, small organic molecules and the like. Such assays for inhibitors and activators include, e.g., expressing TRAC1 protein *in vitro*, in cells, cell extracts, or cell membranes, applying putative modulator compounds, and then determining the functional effects on activity, as described above.

Samples or assays comprising TRAC1 proteins that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative protein activity value of 100%. Inhibition of TRAC1 is achieved when the activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation of TRAC1 is achieved when the activity value relative to the control (untreated with activators) is 110%, more

preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

The term "test compound" or "drug candidate" or "modulator" or grammatical equivalents as used herein describes any molecule, either naturally occurring or synthetic, e.g., protein, oligopeptide (e.g., from about 5 to about 25 amino acids in length, preferably from about 10 to 20 or 12 to 18 amino acids in length, preferably 12, 15, or 18 amino acids in length), small organic molecule, polysaccharide, lipid (e.g., a sphingolipid), fatty acid, polynucleotide, oligonucleotide, etc., to be tested for the capacity to directly or indirectly modulation lymphocyte activation. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a "lead compound") with some desirable property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

A "small organic molecule" refers to an organic molecule, either naturally occurring or synthetic, that has a molecular weight of more than about 50 daltons and less than about 2500 daltons, preferably less than about 2000 daltons, preferably between about 100 to about 1000 daltons, more preferably between about 200 to about 500 daltons.

"Biological sample" include sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. Such samples include blood, sputum, tissue, cultured cells, e.g., primary cultures, explants, and transformed cells, stool, urine, etc. A biological sample is typically obtained from a eukaryotic organism, most preferably a mammal such as a primate e.g., chimpanzee or human; cow; dog; cat; a rodent, e.g., guinea pig, rat, mouse; rabbit; or a bird; reptile; or fish.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region

(e.g., a nucleotide sequence of SEQ ID NO:2), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (*see, e.g.*, NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ or the like). Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nu

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al., supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described

sequence with respect to the expression product, but not with respect to actual probe sequences.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see, e.g.*, Creighton, *Proteins* (1984)).

Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, *see, e.g.*, Alberts *et al.*, *Molecular Biology of the Cell* (3rd ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains, e.g., transmembrane domains, pore domains, cytoplasmic tail domains, ring finger domains, ligase domains, etc. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 15 to 350 amino acids long. Exemplary domains include extracellular domains, transmembrane domains, cytoplasmic domains, ring finger domains, enzymatic activity domains such as ligase domains, etc. Typical domains are made up of sections of lesser organization such as stretches of β-sheet and α-helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

A particular nucleic acid sequence also implicitly encompasses "splice variants." Similarly, a particular protein encoded by a nucleic acid implicitly encompasses any protein encoded by a splice variant of that nucleic acid. "Splice variants," as the name suggests, are products of alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternate polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition.

A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent

and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, e.g., and *Current Protocols in Molecular Biology*, ed. Ausubel, *et al.*

For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase

of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, e.g., in Innis *et al.* (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

"Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'$_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990))

For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity (*see, e.g.*, Kuby, *Immunology* (3rd ed. 1997)). Techniques for the production of single chain antibodies or recombinant antibodies (U.S. Patent 4,946,778, U.S. Patent No. 4,816,567) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized or human antibodies (*see, e.g.*, U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, Marks *et al.*, *Bio/Technology* 10:779-783 (1992); Lonberg *et al.*, *Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild *et al.*, *Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology* 14:826 (1996); and Lonberg & Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995)). Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g.*, McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)). Antibodies can also be made bispecific, i.e., able to recognize two different antigens (*see, e.g.*, WO 93/08829, Traunecker *et al.*, *EMBO J.* 10:3655-3659 (1991); and Suresh *et al.*, *Methods in Enzymology* 121:210 (1986)). Antibodies can also be heteroconjugates, e.g., two covalently joined antibodies, or immunotoxins (*see, e.g.*, U.S. Patent No. 4,676,980 , WO 91/00360; WO 92/200373; and EP 03089).

Methods for humanizing or primatizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of

Winter and co-workers (*see, e.g.*, Jones *et al., Nature* 321:522-525 (1986); Riechmann *et al., Nature* 332:323-327 (1988); Verhoeyen *et al., Science* 239:1534-1536 (1988) and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

In one embodiment, the antibody is conjugated to an "effector" moiety. The effector moiety can be any number of molecules, including labeling moieties such as radioactive labels or fluorescent labels, or can be a therapeutic moiety. In one aspect the antibody modulates the activity of the protein.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to TRAC1 protein as encoded by SEQ ID NO:1, polymorphic variants, alleles, orthologs, and conservatively modified variants, or splice variants, or portions thereof, can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with TRAC1 proteins and not with other proteins. This selection may be achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with

a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

By "therapeutically effective dose" herein is meant a dose that produces effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (*see, e.g.*, Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); and Pickar, *Dosage Calculations* (1999)).

ASSAYS FOR PROTEINS THAT MODULATION T LYMPHOCYTE ACTIVATION

High throughput functional genomics assays can be used to identify modulators of T lymphocyte activation. Such assays can monitor changes in cell surface marker expression, cytokine production, antibody production, proliferation and differentiation, and apoptosis, using either cell lines or primary cells. Typically, the lymphocytes are contacted with a cDNA, a random peptide library (encoded by nucleic acids), or a cyclic peptide library (*see, e.g.*, US Patent No. 6,153,380). The cDNA library can comprise sense, antisense, full length, and truncated cDNAs. The peptide library (optionally cyclic peptides) is encoded by nucleic acids. The lymphocytes are then activated, e.g., by activating the T cell receptor (TCR, also known as CD3), e.g., using antibodies to the receptor. The effect of the cDNA or peptide library on the phenotype of lymphocyte activation is then monitored, using an assay as described above. The effect of the cDNA or peptide can be validated and distinguished from somatic mutations, using, e.g., regulatable expression of the nucleic acid such as expression from a tetracycline promoter. cDNAs and nucleic acids encoding peptides can be rescued using techniques known to those of skill in the art, e.g., using a sequence tag.

Proteins interacting with the peptide or with the protein encoded by the cDNA (e.g., TRAC1) can be isolated using a yeast two-hybrid system, mammalian two hybrid system, or phage display screen, etc. Targets so identified can be further used as bait in these assays to identify additional members of the lymphocyte activation pathway, which members are also targets for drug development (*see, e.g.*, Fields *et al.*, *Nature* 340:245 (1989); Vasavada *et al.*, *Proc. Nat'l Acad. Sci. USA* 88:10686 (1991); Fearon *et*

al., Proc. Nat'l Acad. Sci. USA 89:7958 (1992); Dang *et al., Mol. Cell. Biol.* 11:954 (1991); Chien *et al., Proc. Nat'l Acad. Sci. USA* 9578 (1991); and U.S. Patent Nos. 5,283,173, 5,667,973, 5,468,614, 5,525,490, and 5,637,463).

Suitable T cell lines include Jurkat, HPB-ALL, HSB-2, and PEER, as well as other mature and immature T cell lines and primary T cells known to those of skill in the art. Suitable T cell surface markers include MHC class II, CD2, CD3, CD4, CD5, CD8, CD25, CD28, CD69, CD40L, LFA-1, and ICAM-1 as well as other cell surface markers known to those of skill in the art (*see, e.g.*, Yablonski *et al., Science* 281:413-416 (1998)). Suitable cytokines, for measuring either production or response, include IL-2, IL-4, IL-5, IL-6, IL-10, INF-γ, and TGF-β, as well as their corresponding receptors.

Cell surface markers can be assayed using fluorescently labeled antibodies and FACS. Cell proliferation can be measured using ^{3}H-thymidine or dye inclusion. Apoptosis can be measured using dye inclusion, or by assaying for DNA laddering or increases in intracellular calcium. Cytokine production can be measured using an immunoassay such as ELISA.

cDNA libraries are made from any suitable source, preferably from primary human lymphoid organs such as thymus, spleen, lymph node, and bone marrow. Libraries encoding random peptides are made according to techniques well known to those of skill in the art (*see, e.g.*, U.S. Patent No. 6,153,380, 6,114,111, and 6,180,343). Any suitable vector can be used for the cDNA and peptide libraries, including, e.g., retroviral vectors.

In a preferred embodiment, target proteins that modulate T cell activation are identified using a high throughput cell based assay (using a microtiter plate format) and FACS screening for CD69 cell surface expression (see Example I). cDNA libraries are made from primary lymphocyte organs. These cDNA libraries include, e.g., sense, antisense, full length, and truncated cDNAs. The cDNAs are cloned into a retroviral vector with a tet-regulatable promoter. Jurkat cells are infected with the library, the cells are stimulated with anti-TCR antibodies, and then the cells are sorted using fluorescent antibodies and FACS for CD69 low/CD3+ cells. Cells with the desired phenotype are recovered, expanded, and cloned. A Tet-regulatable phenotype is established to distinguish somatic mutations. The cDNA is rescued. Optionally, the phenotype is validated by assaying for cytokine, e.g., Il-2, production using primary lymphocytes. Optionally, a marker such as GFP can be used to select for retrovirally infected cells.

Using this system, cDNAs encoding TRAC1 were identified as inhibitors of T cell activation.

ISOLATION OF NUCLEIC ACIDS ENCODING TRAC1 FAMILY MEMBERS

This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

TRAC1 nucleic acids, polymorphic variants, orthologs, and alleles that are substantially identical to an amino acid sequence encoded by SEQ ID NO:1, as well as other TRAC1 family members, can be isolated using TRAC1 nucleic acid probes and oligonucleotides under stringent hybridization conditions, by screening libraries. Alternatively, expression libraries can be used to clone TRAC1 protein, polymorphic variants, orthologs, and alleles by detecting expressed homologs immunologically with antisera or purified antibodies made against human TRAC1 or portions thereof.

To make a cDNA library, one should choose a source that is rich in TRAC1 RNA. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (*see, e.g.*, Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*).

For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

An alternative method of isolating TRAC1 nucleic acid and its orthologs, alleles, mutants, polymorphic variants, and conservatively modified variants combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (*see* U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR)

and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of human TRAC1 directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify TRAC1 homologs using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of TRAC1 encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

Gene expression of TRAC1 can also be analyzed by techniques known in the art, e.g., reverse transcription and amplification of mRNA, isolation of total RNA or poly A^+ RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, high density polynucleotide array technology, e.g., and the like.

Nucleic acids encoding TRAC1 protein can be used with high density oligonucleotide array technology (e.g., GeneChip™) to identify TRAC1 protein, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs being identified are linked to modulation of T cell activation, they can be used with GeneChip™ as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al., AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al., Nat. Med.* 2:753-759 (1996); Matson *et al., Anal. Biochem.* 224:110-106 (1995); Lockhart *et al., Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al., Genome Res.* 8:435-448 (1998); Hacia *et al., Nucleic Acids Res.* 26:3865-3866 (1998).

The gene for TRAC1 is typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors.

EXPRESSION IN PROKARYOTES AND EUKARYOTES

To obtain high level expression of a cloned gene, such as those cDNAs encoding TRAC1, one typically subclones TRAC1 into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation.

Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.*, and Ausubel *et al, supra.* Bacterial expression systems for expressing the TRAC1 protein are available in, e.g., *E. coli, Bacillus sp.,* and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one preferred embodiment, retroviral expression systems are used in the present invention.

Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the TRAC1 encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding TRAC1 and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as MBP, GST, and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc. Sequence tags may be included in an expression cassette for nucleic acid rescue. Markers such as fluorescent proteins, green or red fluorescent protein, β-gal, CAT, and the like can be included in the vectors as markers for vector transduction.

Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, retroviral vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Expression of proteins from eukaryotic vectors can be also be regulated using inducible promoters. With inducible promoters, expression levels are tied to the concentration of inducing agents, such as tetracycline or ecdysone, by the incorporation of response elements for these agents into the promoter. Generally, high level expression is obtained from inducible promoters only in the presence of the inducing agent; basal expression levels are minimal.

In one embodiment, the vectors of the invention have a regulatable promoter, e.g., tet-regulated systems and the RU-486 system (*see, e.g.*, Gossen & Bujard, *Proc. Nat'l Acad. Sci. USA* 89:5547 (1992); Oligino *et al., Gene Ther.* 5:491-496 (1998); Wang *et al., Gene Ther.* 4:432-441 (1997); Neering *et al., Blood* 88:1147-1155 (1996); and Rendahl *et al., Nat. Biotechnol.* 16:757-761 (1998)). These impart small molecule control on the expression of the candidate target nucleic acids. This beneficial feature can be used to determine that a desired phenotype is caused by a transfected cDNA rather than a somatic mutation.

Some expression systems have markers that provide gene amplification such as thymidine kinase and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a TRAC1 encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen

such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of TRAC1 protein, which are then purified using standard techniques (*see, e.g.*, Colley *et al., J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, biolistics, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g.*, Sambrook *et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing TRAC1.

After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of TRAC1, which is recovered from the culture using standard techniques identified below.

PURIFICATION OF TRAC1 POLYPEPTIDES

Either naturally occurring or recombinant TRAC1 can be purified for use in functional assays. Naturally occurring TRAC1 can be purified, e.g., from human tissue. Recombinant TRAC1 can be purified from any suitable expression system.

The TRAC1 protein may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra;* and Sambrook *et al., supra*).

A number of procedures can be employed when recombinant TRAC1 protein is being purified. For example, proteins having established molecular adhesion properties can be reversible fused to the TRAC1 protein. With the appropriate ligand,

TRAC1 protein can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, TRAC1 protein could be purified using immunoaffinity columns.

A. Purification of TRAC1 from recombinant bacteria

Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of TRAC1 protein inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g.*, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art. Human TRAC1 proteins are separated from other bacterial proteins by standard separation techniques, e.g., with Ni-NTA agarose resin.

Alternatively, it is possible to purify TRAC1 protein from bacteria periplasm. After lysis of the bacteria, when the TRAC1 protein exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM $MgSO_4$ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

B. Standard protein separation techniques for purifying TRAC1 proteins

Solubility fractionation

Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size differential filtration

The molecular weight of the TRAC1 proteins can be used to isolate it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein

mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column chromatography

The TRAC1 proteins can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

ASSAYS FOR MODULATORS OF TRAC1 PROTEIN

A. Assays

Modulation of a TRAC1 protein, and corresponding modulation of ubiquitination, T lymphocyte activation, and TCR signaling, can be assessed using a variety of *in vitro* and *in vivo* assays, including cell-based models as described above. Such assays can be used to test for inhibitors and activators of TRAC1 protein, and, consequently, inhibitors and activators of ubiquitination or lymphocyte activation. Such modulators of TRAC1 protein, which is involved in ubiquitination, T lymphocyte activation, and TCR signaling, are useful for treating disorders related to ubiquitination and T cell activation. Modulators of TRAC1 protein are tested using either recombinant or naturally occurring TRAC1, preferably human TRAC1. Other mammalian species can be used in the assays of the invention, such as mouse TRAC1 (*see, e.g.*, Figure 1).

Preferably, the TRAC1 protein will have the sequence as encoded by SEQ ID NO:1 or a conservatively modified variant thereof. Alternatively, the TRAC1 protein of the assay will be derived from a eukaryote and include an amino acid subsequence having substantial amino acid sequence identity to SEQ ID NO:1. Generally, the amino acid sequence identity will be at least 60%, preferably at least 65%, 70%, 75%, 80%, 85%, or 90%, most preferably at least 95%.

Measurement of ubiquitination modulation, lymphocyte activation or loss-of-T lymphocyte activation phenotype on TRAC1 protein or cell expressing TRAC1 protein, either recombinant or naturally occurring, can be performed using a variety of assays, *in vitro*, *in vivo*, and *ex vivo*, as described herein. A suitable physical, chemical or phenotypic change that affects activity or binding can be used to assess the influence of a test compound on the polypeptide of this invention. When the functional effects are determined using intact cells or animals, one can also measure a variety of effects such as, in the case of signal transduction, e.g., ligand binding, hormone release, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), changes in cell metabolism such as pH changes, ligase activity, and changes signal transduction such as changes in intracellular second messengers such as Ca^{2+}, IP3, cGMP, or cAMP; as well as changes related to lymphocyte activation and ubiquitination, e.g., protein degradation, ligation, cellular proliferation, cell surface marker expression, e.g., CD69 or CD40L or other T cell surface markers, cytokine production, and apoptosis.

In one preferred embodiment, described herein in Example I, measurement of CD69 activation and FACS sorting is used to identify modulators of T cell activation. In another embodiment, ubiquitin ligase activity is measured as described in WO 01/75145.

In vitro assays

Assays to identify compounds with TRAC1 modulating activity can be performed *in vitro*. Such assays can used full length TRAC1 protein or a variant thereof (*see, e.g.*, SEQ ID NO:1 or a mutant TRAC1 encoded by the nucleotide sequence of SEQ ID NO:4), or a fragment of a TRAC1 protein, such as the ring finger domain or the ligase domain. Purified recombinant or naturally occurring TRAC1 protein or fragments thereof can be used in the *in vitro* methods of the invention. In addition to purified TRAC1 protein, the recombinant or naturally occurring TRAC1 protein can be part of a cellular lysate. As described below, the assay can be either solid state or soluble. Preferably, the protein is bound to a solid support, either covalently or non-covalently. Often, the *in vitro* assays of the invention are ligand binding or ligand affinity assays, either non-competitive or competitive (e.g., binding of E2 ubiquitin conjugating enzymes such as UbcH5, 7, and 8, as well as other members of the ubiquitination pathway such as E1). Other *in vitro* assays include measuring changes in spectroscopic (e.g., fluorescence, absorbance, refractive index), hydrodynamic (e.g., shape), chromatographic, or solubility properties

for the protein. In one embodiment, the *in vitro* assay measures TRAC1 ligase activity (e.g., ubiquitin ligase activity, or ubiquitin-like ligase activity). Ubiquitin ligase activity can be measured as described in WO 01/75145.

In one embodiment, a high throughput binding assay is performed in which the TRAC1 protein or a fragment thereof such as a ring finger or ligase domain is contacted with a potential modulator and incubated for a suitable amount of time. In one embodiment, the potential modulator is bound to a solid support, and the TRAC1 protein is added. In another embodiment, the TRAC1 protein is bound to a solid support. A wide variety of modulators can be used, as described below, including small organic molecules, peptides, antibodies, and TRAC1 ligand analogs. A wide variety of assays can be used to identify TRAC1-modulator binding, including labeled protein-protein binding assays, electrophoretic mobility shifts, immunoassays, enzymatic assays such as phosphorylation assays, and the like. In some cases, the binding of the candidate modulator is determined through the use of competitive binding assays, where interference with binding of a known ligand is measured in the presence of a potential modulator. Either the modulator or the known ligand is bound first, and then the competitor is added. After the TRAC1 protein is washed, interference with binding, either of the potential modulator or of the known ligand, is determined. Often, either the potential modulator or the known ligand is labeled.

In another embodiment, binding of ligands to TRAC1, and disruption of ligand binding to TRAC1, is measured using mammalian or yeast two hybrid assays. In another embodiment, binding of ligands to TRAC1 and disruption of ligand binding to TRAC1 is measured as follows: TRAC1 is incubated with a cellular extract or transfected into a cell, and then TRAC1 is isolated by affinity binding, e.g., with an antibody (*see, e.g.*, Figure 18). Ligands complexed with TRAC1 are washed and identified using mass spectroscopy. Such ligand binding assays are useful for identifying modulators of TRAC1 complexes, e.g., TRAC1 complexes with other molecules of the ubiquitination pathway such as E1 and E2 molecules.

Cell-based *in vivo* assays

In another embodiment, TRAC1 protein is expressed in a cell, and functional, e.g., physical and chemical or phenotypic, changes are assayed to identify TRAC1 and lymphocyte activation modulators. Cells expressing TRAC1 proteins can also be used in binding assays. Any suitable functional effect can be measured, as

described herein. For example, ligand binding, cell surface marker expression, cellular proliferation, apoptosis, cytokine production, and ligase activity, are all suitable assays to identify potential modulators using a cell based system. Suitable cells for such cell based assays include both primary cells and cell lines, as described herein. In one embodiment, lymphocytes, either primary or cultured, are used in the assays of the invention The TRAC1 protein can be naturally occurring or recombinant.

As described above, in one embodiment, lymphocyte activation is measured by contacting T cells comprising a TRAC1 target with a potential modulator and activating the cells with an anti-TCR antibody. Modulation of T cell activation is identified by screening for cell surface marker expression, e.g., CD69 or CD40L expression levels, using fluorescent antibodies and FACS sorting.

In another embodiment, cellular proliferation or apoptosis can be measured using ^{3}H-thymidine incorporation or dye inclusion. Cytokine production can be measured using an immunoassay such as an ELISA.

In another embodiment, cellular TRAC1 polypeptide levels are determined by measuring the level of protein or mRNA. The level of TRAC1 protein or proteins related to TRAC1 signal transduction are measured using immunoassays such as western blotting, ELISA and the like with an antibody that selectively binds to the TRAC1 polypeptide or a fragment thereof. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization, RNAse protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

Signal transduction related to TCR signaling can also be measured. Activated or inhibited TCR signaling will alter the properties of target enzymes, second messengers, channels, and other effector proteins. The examples include the activation of cGMP phosphodiesterase, adenylate cyclase, phospholipase C, IP3, and modulation of diverse channels. Downstream consequences can also be examined such as generation of diacyl glycerol and IP3 by phospholipase C, and in turn, for calcium mobilization by IP3. For example, changes in Ca^{2+} levels are optionally measured using fluorescent Ca^{2+} indicator dyes and fluorometric imaging.

Alternatively, TRAC1 expression can be measured using a reporter gene system. Such a system can be devised using a TRAC1 protein promoter operably linked to a reporter gene such as chloramphenicol acetyltransferase, firefly luciferase, bacterial

luciferase, β-galactosidase and alkaline phosphatase. Furthermore, the protein of interest can be used as an indirect reporter via attachment to a second reporter such as red or green fluorescent protein (*see, e.g.*, Mistili & Spector, *Nature Biotechnology* 15:961-964 (1997)). The reporter construct is typically transfected into a cell. After treatment with a potential modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

Animal models

Animal models of lymphocyte activation also find use in screening for modulators of ubiquitination or lymphocyte activation. Similarly, transgenic animal technology including gene knockout technology, for example as a result of homologous recombination with an appropriate gene targeting vector, or gene overexpression, will result in the absence or increased expression of the TRAC1 protein. When desired, tissue-specific expression or knockout of the TRAC1 protein may be necessary. Transgenic animals generated by such methods find use as animal models of ubiquitination and lymphocyte activation and are additionally useful in screening for modulators of lymphocyte activation.

Knock-out cells and transgenic mice can be made by insertion of a marker gene or other heterologous gene into the endogenous TRAC1 gene site in the mouse genome via homologous recombination. Such mice can also be made by substituting the endogenous TRAC1 with a mutated version of TRAC1, or by mutating the endogenous TRAC1, e.g., by exposure to carcinogens.

A DNA construct is introduced into the nuclei of embryonic stem cells. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is re-implanted into a recipient female. Some of these embryos develop into chimeric mice that possess germ cells partially derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (*see, e.g.*, Capecchi *et al., Science* 244:1288 (1989)). Chimeric targeted mice can be derived according to Hogan *et al.*, *Manipulating the Mouse Embryo*: *A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed., IRL Press, Washington, D.C., (1987).

B. Modulators

The compounds tested as modulators of TRAC1 protein can be any small organic molecule, or a biological entity, such as a protein, e.g., an antibody or peptide, a sugar, a nucleic acid, e.g., an antisense oligonucleotide, RNAi, or a ribozyme, or a lipid. Alternatively, modulators can be genetically altered versions of a TRAC1 protein. Typically, test compounds will be small organic molecules, peptides, lipids, and lipid analogs.

Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

In one preferred embodiment, high throughput screening methods involve providing a combinatorial small organic molecule or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are

not limited to, peptide libraries (*see, e.g.,* U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al., Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al., J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al., J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al., J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al., Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al., J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.,* U.S. Patent 5,539,083), antibody libraries (*see, e.g.,* Vaughn *et al., Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see, e.g.,* Liang *et al., Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.,* benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.,* 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.,* ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

C. Solid state and soluble high throughput assays

In one embodiment the invention provides soluble assays using a TRAC1 protein or a fragment thereof such as the ring finger or ligase domain, or a cell or tissue expressing a TRAC1 protein, either naturally occurring or recombinant. In another embodiment, the invention provides solid phase based *in vitro* assays in a high throughput

format, where the TRAC1 protein or fragment thereof, such as the ring finger or ligase domain, is attached to a solid phase substrate. Any one of the assays described herein can be adapted for high throughput screening, e.g., ligand binding, cellular proliferation, cell surface marker flux, e.g., CD-69 screening, ligase activity, second messenger flux, e.g., Ca^{2+}, IP3, cGMP, or cAMP, cytokine production, etc. In one preferred embodiment, the cell-based system using CD-69 modulation and FACS assays is used in a high throughput format for identifying modulators of TRAC1 proteins, and therefore modulators of T cell activation. In another preferred embodiment, the ligase domain or the ring finger domain of TRAC1 is used in high throughput *in vitro* binding assays for modulators.

In the high throughput assays of the invention, either soluble or solid state, it is possible to screen up to several thousand different modulators or ligands in a single day. This methodology can be used for TRAC1 proteins *in vitro*, or for cell-based or membrane-based assays comprising a TRAC1 protein. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay many plates per day; assay screens for up to about 6,000, 20,000, 50,000, or more than 100,000 different compounds are possible using the integrated systems of the invention.

For a solid state reaction, the protein of interest or a fragment thereof, e.g., an enzymatic or ring finger domain, or a cell or membrane comprising the protein of interest or a fragment thereof as part of a fusion protein can be bound to the solid state component, directly or indirectly, via covalent or non covalent linkage e.g., via a tag. The tag can be any of a variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid support, and the tagged molecule of interest is attached to the solid support by interaction of the tag and the tag binder.

A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, etc.) Antibodies to molecules with natural binders such as biotin are also widely available and appropriate tag binders; *see,* SIGMA Immunochemicals 1998 catalogue SIGMA, St. Louis MO).

Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair. Thousands of specific antibodies are commercially available and many additional antibodies are described in the literature. For example, in one common configuration, the tag is a first antibody and the tag binder is a second antibody which recognizes the first antibody. In addition to antibody-antigen interactions, receptor-ligand interactions are also appropriate as tag and tag-binder pairs. For example, agonists and antagonists of cell membrane receptors (e.g., cell receptor-ligand interactions such as transferrin, c-kit, viral receptor ligands, cytokine receptors, chemokine receptors, interleukin receptors, immunoglobulin receptors and antibodies, the cadherein family, the integrin family, the selectin family, and the like; *see, e.g.,* Pigott & Power, *The Adhesion Molecule Facts Book I* (1993). Similarly, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), intracellular receptors (e.g. which mediate the effects of various small ligands, including steroids, thyroid hormone, retinoids and vitamin D; peptides), drugs, lectins, sugars, nucleic acids (both linear and cyclic polymer configurations), oligosaccharides, proteins, phospholipids and antibodies can all interact with various cell receptors.

Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly gly sequences of between about 5 and 200 amino acids. Such flexible linkers are known to persons of skill in the art. For example, poly(ethelyne glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent which fixes a chemical group to the surface which is reactive with a portion of the tag binder. For example, groups which are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of

such solid phase biopolymer arrays is well described in the literature. *See, e.g.*, Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963) (describing solid phase synthesis of, e.g., peptides); Geysen *et al., J. Immun. Meth.* 102:259-274 (1987) (describing synthesis of solid phase components on pins); Frank & Doring, *Tetrahedron* 44:60316040 (1988) (describing synthesis of various peptide sequences on cellulose disks); Fodor *et al., Science*, 251:767-777 (1991); Sheldon *et al., Clinical Chemistry* 39(4):718-719 (1993); and Kozal *et al., Nature Medicine* 2(7):753759 (1996) (all describing arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by UV radiation, and the like.

IMMUNOLOGICAL DETECTION OF TRAC1 POLYPEPTIDES

In addition to the detection of TRAC1 gene and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect TRAC1 proteins of the invention. Such assays are useful for screening for modulators of TRAC1 and lymphocyte activation, as well as for therapeutic and diagnostic applications. Immunoassays can be used to qualitatively or quantitatively analyze TRAC1 protein. A general overview of the applicable technology can be found in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988).

A. Production of antibodies

Methods of producing polyclonal and monoclonal antibodies that react specifically with the TRAC1 proteins are known to those of skill in the art (*see, e.g.*, Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g.*, Huse *et al., Science* 246:1275-1281 (1989); Ward *et al., Nature* 341:544-546 (1989)).

A number of immunogens comprising portions of TRAC1 protein may be used to produce antibodies specifically reactive with TRAC1 protein. For example, recombinant TRAC1 protein or an antigenic fragment thereof, can be isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic

cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (e.g., BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see,* Harlow & Lane, *supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see,* Kohler & Milstein, *Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse, *et al.*, *Science* 246:1275-1281 (1989).

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against non- TRAC1 proteins, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a K_d of at least

about 0.1 mM, more usually at least about 1 μM, preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better. Antibodies specific only for a particular PAK family member, such as TRAC1, or a particular TRAC1 ortholog, such as human TRAC1, can also be made, by subtracting out other cross-reacting PAK family members or orthologs from a species such as a non-human mammal. In this manner, antibodies that bind only to a particular PAK protein or ortholog may be obtained.

Once the specific antibodies against TRAC1 protein are available, the protein can be detected by a variety of immunoassay methods. In addition, the antibody can be used therapeutically as a TRAC1 modulators. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay (Maggio, ed., 1980); and Harlow & Lane, *supra*.

B. Immunological binding assays

TRAC1 protein can be detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7th ed. 1991). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein or antigen of choice (in this case the TRAC1 protein or antigenic subsequence thereof). The antibody (e.g., anti- TRAC1) may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may itself be one of the moieties comprising the antibody/antigen complex. Thus, the labeling agent may be a labeled TRAC1 or a labeled anti- TRAC1 antibody. Alternatively, the labeling agent may be a third moiety, such a secondary antibody, that specifically binds to the antibody/ TRAC1 complex (a secondary antibody is typically specific to antibodies of the species from which the first antibody is derived). Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, e.g.*, Kronval *et al.*, *J.*

Immunol. 111:1401-1406 (1973); Akerstrom *et al.*, *J. Immunol.* 135:2589-2542 (1985)). The labeling agent can be modified with a detectable moiety, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, optionally from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

Non-competitive assay formats

Immunoassays for detecting TRAC1 in samples may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly measured. In one preferred "sandwich" assay, for example, the anti-TRAC1 antibodies can be bound directly to a solid substrate on which they are immobilized. These immobilized antibodies then capture TRAC1 present in the test sample. TRAC1 proteins thus immobilized are then bound by a labeling agent, such as a second TRAC1 antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second or third antibody is typically modified with a detectable moiety, such as biotin, to which another molecule specifically binds, e.g., streptavidin, to provide a detectable moiety.

Competitive assay formats

In competitive assays, the amount of TRAC1 protein present in the sample is measured indirectly by measuring the amount of a known, added (exogenous) TRAC1 protein displaced (competed away) from an anti- TRAC1 antibody by the unknown TRAC1 protein present in a sample. In one competitive assay, a known amount of TRAC1 protein is added to a sample and the sample is then contacted with an antibody that specifically binds to TRAC1 protein. The amount of exogenous TRAC1 protein bound to the antibody is inversely proportional to the concentration of TRAC1 protein

present in the sample. In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of TRAC1 protein bound to the antibody may be determined either by measuring the amount of TRAC1 present in TRAC1 protein/antibody complex, or alternatively by measuring the amount of remaining uncomplexed protein. The amount of TRAC1 protein may be detected by providing a labeled TRAC1 molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay the known TRAC1 protein is immobilized on a solid substrate. A known amount of anti- TRAC1 antibody is added to the sample, and the sample is then contacted with the immobilized TRAC1. The amount of anti-TRAC1 antibody bound to the known immobilized TRAC1 is inversely proportional to the amount of TRAC1 protein present in the sample. Again, the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

Cross-reactivity determinations

Immunoassays in the competitive binding format can also be used for crossreactivity determinations. For example, a TRAC1 protein can be immobilized to a solid support. Proteins (e.g., TRAC1 and homologs) are added to the assay that compete for binding of the antisera to the immobilized antigen. The ability of the added proteins to compete for binding of the antisera to the immobilized protein is compared to the ability of the TRAC1 protein to compete with itself. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with each of the added proteins listed above are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the added considered proteins, e.g., distantly related homologs.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein, thought to be perhaps an allele or polymorphic variant of a TRAC1 protein, to the immunogen protein. In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein

required to inhibit 50% of binding is less than 10 times the amount of the TRAC1 protein that is required to inhibit 50% of binding, then the second protein is said to specifically bind to the polyclonal antibodies generated to TRAC1 immunogen.

Other assay formats

Western blot (immunoblot) analysis is used to detect and quantify the presence of TRAC1 in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind TRAC1. The anti- TRAC1 antibodies specifically bind to the TRAC1 on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (e.g., labeled sheep anti-mouse antibodies) that specifically bind to the anti-TRAC1 antibodies.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (e.g., antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see* Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34-41 (1986)).

Reduction of non-specific binding

One of skill in the art will appreciate that it is often desirable to minimize non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this technique involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-

developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS™), fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to another molecules (e.g., streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize TRAC1 protein, or secondary antibodies that recognize anti- TRAC1.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be

detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

CELLULAR TRANSFECTION AND GENE THERAPY

The present invention provides the nucleic acids of TRAC1 protein for the transfection of cells *in vitro* and *in vivo*. These nucleic acids can be inserted into any of a number of well-known vectors for the transfection of target cells and organisms as described below. The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The nucleic acid, under the control of a promoter, then expresses a TRAC1 protein of the present invention, thereby mitigating the effects of absent, partial inactivation, or abnormal expression of a TRAC1 gene, particularly as it relates to T cell activation. The compositions are administered to a patient in an amount sufficient to elicit a therapeutic response in the patient. An amount adequate to accomplish this is defined as "therapeutically effective dose or amount."

Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and other diseases in a number of contexts. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies (for a review of gene therapy procedures, *see* Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, TIBTECH 11:211-217 (1993); Mitani & Caskey, TIBTECH 11:162-166 (1993); Mulligan, *Science* 926-932 (1993); Dillon, TIBTECH 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1998); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et*

al., in *Current Topics in Microbiology and Immunology* (Doerfler & Böhm eds., 1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994)).

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*see, e.g.*, *Remington's Pharmaceutical Sciences*, 17th ed., 1989). Administration can be in any convenient manner, e.g., by injection, oral administration, inhalation, transdermal application, or rectal administration.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

The compound of choice, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-

aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of commends can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by nucleic acids for *ex vivo* therapy can also be administered intravenously or parenterally as described above.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular patient.

In determining the effective amount of the vector to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant expression of the TRAC1 protein, the physician evaluates circulating plasma levels of the vector, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 μg to 100 μg for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

For administration, compounds and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: Identification of genes involved in modulation of T cell activation

A. Introduction

In this study, an approach to identify new targets for immune suppressive drugs is provided. It is known that following T cell activation, expression of numerous cell surface markers such as CD25, CD69, and CD40L are upregulated. CD69 has been shown to be an early activation marker in T, B, and NK cells. CD69 is a disulfide-linked dimer. It is not expressed in resting lymphocytes but appears on T, B and NK cells after activation in vitro. Its relevance as a TCR signaling outcome has been validated using T cell deficient in certain key signaling molecules such as LAT and SLP76 (Yablonski, *supra*). Furthermore, re-introducing SLP76 to the deficient cells results in restoration of CD69 expression. CD69 upregulation was therefore to be used to monitor TCR signal transduction. The rationale of the functional genomics screen was then to identify cell clones whose CD69 upregulation was repressed following introduction of a retroviral cDNA library. The library members conferring such repression would then represent immune modulators that function to block TCR signal transduction.

B. Results

Several T cell lines, including Jurkat, HPB-ALL, HSB-2 and PEER were tested for the presence of surface CD3, CD25, CD28, CD40L, CD69, CD95, and CD95L. Those that express CD3 were cultured with anti-CD3 or anti-TCR to crosslink the TCR and examined for the upregulation of CD69. Jurkat T cell line was selected for its ability to upregulate CD69 in response to crosslinking of their TCR with a kinetics mimicking that of primary T lymphocytes (data not shown). The population of Jurkat cells was sorted for low basal and highly inducible CD69 expression following anti-TCR stimulation. Clone 4D9 was selected because CD69 in this clone was uniformly and strongly induced following TCR stimulation in 24 hours.

In order to regulate the expression of the retroviral library, the Tet-Off system was used. Basically, cDNA inserts in the retroviral library were cloned behind the tetracycline regulatory element (TRE) and the minimal promoter of TK. Transcription of the cDNA inserts were then dependent on the presence of tetracycline-controlled trans-activator (tTA), a fusion of Tet repression protein and the VP16 activation domain, and the absence of tetracyaline or its derivatives such as doxycycline (Dox). To shut off the cDNA expression, one can simply add doxycycline in the medium. To obtain a Jurkat clone stably expresses tTA, retroviral LTR-driven tTA was introduced in conjunction

with a TRE-dependent reporter construct, namely TRA-Lyt2. Through sorting of Lyt2 positive cells in the absence of Dox and Lyt2 negative cells in the presence of Dox , coupled with clonal evaluation, a derivative of Jurkat clone 4D9 was obtained, called 4D9#32, that showed the best Dox regulation of Lyt2 expression.

Positive controls: ZAP70 is a positive regulator of T cell activation. A kinase-inactivated (KI) ZAP70 and a truncated ZAP70 (SH2 N+ C) were subcloned into the retroviral vector under TRE control. ZAP70 SH2 (N+ C) and ZAP70 KI both inhibited TCR-induced CD69 expression. Consistent with the published report on dominant negative forms of ZAP70 on NFAT activity, the truncated protein is also a more potent inhibitor of CD69 induction. In addition, the higher protein expression, as shown by adjusting GFP-gating, the stronger the inhibition was. When one puts the marker M1 at bottom 1% of the uninfected cells, one has a 40% likelihood of obtaining cells whose phenotype resembled that of ZAP70 SH2 (N+C). This translates into a 40:1 enrichment of the desired phenotype.

The CD69 inhibitory phenotype is dependent on expression of dominant negative forms of ZAP70. When Dox was added for 7 days before TCR was stimulated, there was no inhibition of CD69 expression. Analysis of cellular phenotype by FACS of GFP, which was produced from the bi-cistronic mRNA ZAP70 SH2 (N+C)-IRES-GFP, revealed a lack of GFP+ cells. The lack of ZAP70 SH2 (N+C) expression in the presence of Dox was confirmed by Western.

Screening for cells lacking CD69 upregulation: Jurkat 4D9#32 cells were infected with cDNA libraries made form primary human lymphoid organs such as thymus, spleen, lymph node and bone marrow. The library complexity was $5x10^7$ and was built on the TRE vector. A total of $7.1x10^8$ cells were screened with an infection rate of 52%, as judged by parallel infection of the same cells with TRA-dsGFP (data not shown). After infection, the cells will be stimulated with the anti-TCR antibody C305 for overnight and sorted for CD69 low and CD3+ phenotype by FACS. If the sorting gate was set to include the bottom 3% cells based on the single parameter of CD69 level, 2/3 cells in the sorting gate lacked TCR/CD3 complex, which explained their refractory to stimulation. The second parameter of CD3 expression was then incorporated. Even though there was a significant reduction of CD3/TCR complex on the surface following receptor-mediated internalization, the CD3- population was still distinguishable from the

CD3+ population. The resulting sort gate contained 1% of the total cells, which translated into a 100-fold enrichment based on cell numbers. The recovered cells with CD69 low CD3+ phenotype were allowed to rest in complete medium for 5 days before being stimulated again for a new round of sorting. In subsequent round of sortings, the sort gate was always maintained to contain the equivalent of 1% of the unsorted control population. Obvious enrichment was achieved after 3 rounds of reiterative sorting. Cells with the desired phenotype increased from 1% to 22.3%. In addition, the overall population's geometric mean for CD69 was also reduced.

In order to ascertain that the phenotype was due to expression of the cDNA library rather than entirely due to spontaneous or retroviral insertion-mediated somatic mutation, the cells recovered after the third round of sorting were split into two halves. One half of the cells were grown in the absence of Dox while the other half in the presence of Dox. A week later, CD69 expression was compared following anti-TCR stimulation. There was a significant numbers of cells (11%) whose CD69 repression was lost in the presence of Dox, suggesting that the CD69 inhibition phenotype was indeed caused by the expression of library members. Single cell clones in conjunction with the fourth round of CD69 low CD3+ sorting (LLLL) were deposited.

In order to reduce the number of cells whose phenotype was not Dox-regulatable, the half of the cells grown in the presence of Dox were subjected to a fourth round of sorting for enrichment of CD69 high phenotype (LLLH). The cells recovered from LLLH sort were cultured in the absence of Dox for subsequence sorting and single cell cloning of CD69 low CD3+ phenotypes.

Dox regulation of CD69 expression was expressed as the ratio of geometric mean fluorescent intensity (GMFI) in the presence of Dox over that in the absence of Dox. In uninfected cells, Dox had limited effect on the induction of CD69 expression so that the ratio of GMFI (+Dox)/GMFI (-Dox) remained to be 1.00+/-0.25. The 2x standard deviation was therefore used as a cut-off criterion and clones with a ratio above 1.5 were regarded as Dox-regulated clones.

RNA samples were prepared from clones with Dox-regulatable phenotypes. Using primers specific for the vector sequence flanking the cDNA library insert, the cDNA insert of selected clones were captured by RT-PCR. Most clones generated only on DNA band, whereas a few clones generated two or more bands. Sequencing analysis revealed that the additional bands were caused by double or multiple insertions.

Characterization of proteins involved in T cell activation: Known TCR regulators such as Lck, ZAP70, PLCγ1 and Raf were obtained. In addition, the BCR regulator SYK was also uncovered. Molecules previously not associated with TCR activation, such as TRAC1, were also identified using this screen.

Lck is a non-receptor protein tyrosine kinase. Its role in T cell development and activation has been widely documented. So far, dominant negative form of Lck has no been reported. Our discovery that over expression of the kinase-truncated form of Lck caused inhibition of CD69, similar to the phenotype of Jurkat somatic mutant lacking Lck, suggests that kinase deletion of Lck could also work as a dominant negative form of Lck.

The two ZAP70 hits ended at aa 262 and 269, respectively. They both missed the catalytic domain. The deletions are very close to the positive control for the screen, ZAP70 SH2 (N+C), which ended at aa 276. Since ZAP70 SH2 (N+C) was shown to be a dominant negative protein, it appears that the two ZAP70 hits also behaved as dominant negative proteins of ZAP70.

SYK is a non-receptor tyrosine kinase belonging to the SYK/ZAP70 family of kinases. Since it has also been shown that the lack of SYK expression in Jurkat cells did not appear to significantly alter the TCR-mediated responses compared with Jurkat clones expressing SYK, it appears that the SYK hit obtained from our screen worked mainly to block ZAP70 function. SYK's similarity to ZAP70 and its ability to associate with phosphorylated TCR zeta chains also support this notion.

PLCγ1 plays a crucial role in coupling T cell receptor ligation to IL-2 gene expression in activated T lymphocytes. TCR engagement leads to rapid tyrosine phosphorylation and activation of PLCγ1. The activated enzyme converts phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,3,5-trisphosphate ((IP3) and diacylglycerol (DAG). IP3 triggers intracellular Ca2+ increase and DAG is a potent activator of protein kinase C (PKC). PLCγ1 has a split catalytic domain comprised of conserved X and Y subdomains. Single point mutation in the catalytic X box completely abolished the enzyme activity and also blocked IL-2 reporter gene expression when introduced into PLCγ-deficient Jurkat cells. Our hit contained the PH domain and the N and C terminal SH2 domains of PLCγ1. Significantly this hit also deleted the crucial tyrosine Y783 between the SH2 and SH3 domains. It was reported that Y783 was essential for coupling of TCR stimulation to IL-2 promoter activation and that mutation

of Y783 to F (phenylalanine) generated a very potent dominant negative form of PLCγ1. Indeed, the original clone encoding the PLCγ1hit had the highest Dox +/- ratio for CD69 expression among all clones from the cDNA screen, indicating the strong repression of CD69 induction by the hit as well as the total de-repression in the absence of the hit. When introduced to naïve Jurkat cells, this fragment caused severe block of TCR-induced CD69 expression.

Raf is a MAP kinase kinase kinase. It interacts with Ras and leads to activation of the MAP kinase pathway. The Raf hit obtained also had a truncation of the kinase domain, creating a dominant negative form of the kinase. Other signaling molecules known to involve in TCR pathway were also discovered in our screen. They included PAG , CSK, SHP-1 and nucleolin.

TRAC1 is a ubiquitin kinase and a member of the ring finger family of proteins. A truncated TRAC1 cDNA was isolated as a functional hit using the T cell CD69 assay described herein. Overexpression of the truncated form of TRAC1 (SEQ ID NO:4), in Jurkat T cells resulted in marked inhibition of TCR mediated CD69 upregulation. The inhibitory effect by overexpressing the truncated TRAC1 was specific to T cells, since it failed to affect the PCR-induced CD69 activation in BJAB cells. TRAC1 is also involved in the TCR signaling pathway, as mutant TRAC1 blocks TCR-induced calcium influx.

Function in primary T lymphocytes: The relevance of the CD69 screen hits to physiological function of T cells was investigated in primary T lymphocytes. The hit was subcloned into a retroviral vector under a constitutively active promoter, followed by IRES-GFP. A protocol was also developed to couple successful retroviral infection to subsequence T cell activation. Primary T lymphocytes are at the quiescent stage when isolated from healthy donors. In order to be infected by retrovirus, primary lymphocytes need to be activated to progress in cell cycle. Fresh peripheral blood lymphocytes (PBL) contained typically T cells and B cells. The combined CD4+ and CD8+ cells represented total T cell percentage, which was 81% in this particular donor. The remaining 19% CD4-CD8- cells were B cells as stained by CD19 (data not shown). Upon culturing on anti-CD3 and anti-CD28 coated dishes, primary T lymphocytes were expanded and primary B cells and other cell types gradually died off in the culture. After infection, the culture contained virtually all T cells. Furthermore, primary T lymphocytes were successfully infected by retroviruses. As seen with Jurkat cells (data not shown), GFP

translated by way of IRES was not as abundant as GFP translated using the conventional Kozak sequence (comparing GFP geometric mean from CRU5-IRES-GFP and CRU5-GFP). Nevertheless the percentage infection remained similar. Insertion of a gene in front of IRES-GFP further reduced the expression level of GFP, which was observed with cell lines (data not shown) and here primary T lymphocytes. After allowing cells to rest following infection, FACS sorted cells were divided into two populations: GFP- and GFP+. The sorted cells were immediately put into culture. Anti-CD3 alone did not induce IL-2 production. This observation was consistent with previous report on freshly isolated primary T lymphocytes and confirmed the notion that prior culture and retroviral infection did not damage the physiological properties of these primary T lymphocytes. Addition of anti-CD28 in conjunction with anti-CD3 led to robust IL-2 production with vector-infected cells and the GFP- population of LckDN and PLCγ1DN-infected cells. The GFP+ cell population from LckDN and PLCγ1DN-infected cells, however, were severed impaired in IL-2 production. As expect, the defect caused by LckDN and PLCγ1DN can be completely rescued by stimulation using PMA and ionomycin. Taken together, these results showed that Lck and PLCγ1 plays a role in IL-2 production from primary T lymphocytes, consistently with their involvement membrane proximal signaling events of T cell activation. These results also demonstrated a successful system to quickly validate hits from our functional genetic screens in primary cells.

Use of CD69 upregulation in drug screening: The discovery of important immune regulatory molecules from the T cell activation-induced CD69 upregulation validated the relevance of this cell-based assay. Essentially such a cell-based assay offers the opportunity to discover inhibitors of multiple targets such as Lck, ZAP70, PLCγ1 and TRAC1. It is the equivalent of multiplexing enzymatic assays with the additional advantage of cell permeability of compounds. It may even be possible to identify novel compounds that block adaptor protein functions. Towards this end, the FACS assay of cell surface CD69 expression was converted to a micro-titer plate based assay.

In conclusion, the strategy presented in this study demonstrates a successful approach to discover and validate important immune regulators on a genome-wide scale. This approach, which requires no prior sequence information, provides a tool for functional cloning of regulators in numerous signal transduction pathways. For

example, B cell activation-induced CD69 expression, IL-4-induced IgE class switch and TNF -induced NF-kB reporter gene expression are all amendable to the genetic perturbation following introduction of retroviral cDNA libraries. The outlined strategy is less biased compared to forced introduction of a handful of signaling molecules discovered in other context such as growth factor signal transduction. It also opens the door for discovering peptide inhibitors of immune modulatory proteins by screening random peptide libraries expressed from the retroviral vector.

C. Methods

Cell culture: Human Jurkat T cells (clone N) were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone), penicillin and streptamycin. Phoenix A cells were grown in DMEM supplemented with 10% fetal calf serum, penicillin and streptamycin. To produce the tTA-Jurkat cell line, Jurkat cells were infected with a retroviral construct which constitutively expresses the tetracycline transactivator protein and a reporter construct which expresses LyT2 driven by a tetracycline responsive element (TRE). The tTA-Jurkat cell population was optimized by sorting multiple sounds for high TRE-dependent expression of LyT2 in the absence of Dox and strong repression of LyT2 expression in the presence Dox. The cells were also sorted for maximal anti-TCR induced expression of CD69. Doxycycline was used at a final concentration of 10 ng/ml for at least 6 days to downregulate expression of cDNAs from the TRE promoter.

Transfection and infection: Phoenix A packaging cells were transfected with retroviral vectors using calcium phosphate for 6 hours as standard protocols. After 24 hours, supernatant was replaced with complete RPMI medium and virus was allowed to accumulate for an additional 24 hours. Viral supernatant was collected, filtered through a 0.2 μM filter and mixed with Jurkat cells at a density of 2.5×10^5 cells/ml. Cells were spun at room temperature for 3 hours at 3000 rpm, followed by overnight incubation at 37°C. Transfection and infection efficiencies were monitored by GFP expression and functional analysis was carried out 2-4 days after infection.

Libraries: RNA extracted from human lymph node, thymus, spleen and bone marrow was used to produce two cDNA libraries; one random primed and

directionally cloned and the second non-directionally cloned and provided with 3 exogenous ATG in 3 frames. cDNAs were cloned into the pTRA-exs vector giving robust doxycycline–regulable transcription of cDNAs from the TRE promoter. The total combined library complexity was 5 x 10^7 independent clones.

Stimulation: For CD69 upregulation experiments, tTA-Jurkat cells were split to 2.5 x 10^5 cells/ml 24 hours prior to stimulation. Cells were spun and resuspended at 5 x 10^5 cells/ml in fresh complete RPMI medium in the presence of 100 ng/ml C305 (anti-Jurkat clonotypic TCR) or 5 ng/ml PMA hybridoma supernatant for 20-26 hours at 37°C, and then assayed for surface CD69 expression.

Cell surface marker analysis: Jurkat-N cells were stained with an APC-conjugated mouse monoclonal anti-human CD69 antibody (Caltag) at 4°C for 20 minutes and analyzed using a Facscalibur instrument (Becton Dickinson) with Cellquest software. Cell sorts were performed on a MoFlo (Cytomation).

cDNA screen: Phoenix A packaging cells were transfected with a mixture of the two tTA regulated retroviral pTRA-exs cDNA libraries. Supernatant containing packaged viral particles was used to infect tTA-Jurkat cells with an efficiency of ~85%. After 4 days of cDNA expression, library infected cells were stimulated with 0.3 μg/ml C305 for 20-26 hours, stained with APC-conjugated anti-CD69, and lowest CD69-expressing cells still expressing CD3 ($CD69^{low}CD3^{+}$) were isolated using a fluorescence activated cell sorter. Sorting was repeated over multiple rounds with a 6-day rest period between stimulations until the population was significantly enriched for non-responders. Single cells were deposited from 4 separate rounds of sorting. Cell clones were expanded in the presence and absence of Dox, stimulated and analyzed for CD69 upregulation.

Isolation of cDNA inserts: PCR primers were designed to amplify cDNA inserts from both libraries and did not amplify Lyt2 that was also under TRE regulation. The primers used contained flanking BstXI sites for subsequent cloning to pTRA-IRES-GFP vector. RT-PCR cloning was achieved with kits from Clontech or Life Technologies. The gel-purified RT-PCR products were submitted for sequencing directly and simultaneously digested for subcloning. Dominant negative ZAP70 (KI) and

ZAP70SH2 (N+C) as well as selected hits from cDNA screens were subcloned to the retroviral pTRA-IRES-GFP vector. Selected hits from cDNA screens were also subcloned to CRU5-IRES-GFP for infection of human primary T lymphocytes and examination of IL-2 production.

Example 2: Yeast Two hybrid assays

As shown in Figure 18, using yeast two hybrid assays and biochemical characterization, wild type TRAC1 was shown to bind to E2 ubiquitin ligase conjugating enzymes such as Ubc5 and Ubc7.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.